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Transcription Mapping as a Tool in Phage Genomics: The Case of the Temperate *Streptococcus thermophilus* Phage Sfi21

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For the lytic growth cycle of the temperate *cos*-site *Streptococcus thermophilus* phage Sfi21 a transcription map was developed on the basis of systematic Northern blot hybridizations. All deduced 5′ ends were confirmed by primer extension analysis. Three time classes of transcripts were observed. Early transcripts were identified in four different genome regions. One prominent early mRNA of 4.8 kb length covered a group of 12 genes located between the origin of replication and the *cos*-site. Two short early mRNAs represented a single gene from the direct vicinity of the *cos*-site and the superinfection immunity gene from the lysogeny module, respectively. A fourth early transcript covered a group of four genes located between the lysin and the integrase gene. Middle transcripts of 2.1 and 5.8 kb length covered *cro*-like and *ant*-like repressor genes and the DNA replication module, respectively. Four types of late transcripts were identified. The transcripts covered the likely DNA packaging genes, the head morphogenesis module plus the major tail gene, the remainder of the tail genes, and the putative tail fiber plus lysis genes, respectively. Only the transcript from the head morphogenesis genes yielded defined late mRNA species. The transcription map concurred with most of the *in silico* predictions for the genome organization of phage Sfi21 except for the separation of the DNA replication module from a possible transcription regulation module. Most 5′ ends of the transcripts determined in primer-extension experiments were not preceded by a consensus promoter sequence. The involvement of phage-encoded regulators for middle and late transcription was suggested by chloramphenicol-inhibition experiments.

INTRODUCTION

Streptococcus thermophilus phages are a major cause of fermentation failures in the dairy industry (Brüssow, 1999). Due to this economic importance, *S. thermophilus* bacteriophage became one of the most carefully sequenced phage groups (Brüssow and Desiere, 2001). All *S. thermophilus* phages belong to the *Siphoviridae* family of tailed phages (*Caudovirales*). Closely related temperate and virulent *S. thermophilus* phages were described (Brüssow, 2001) that shared sequence similarity not only with phages from other important dairy starters (*Lactococcus lactis*, *Lactobacillus* spp.) (Desiere *et al.,* 2001a), but also from important human pathogens such as *S. pyogenes* (Desiere *et al.,* 2001b). Therefore *S. thermophilus* phages were chosen in our laboratory as reference phages for comparative phage genomics. However, simple database searches with phage sequences were of limited use in the elucidation of the phage genome organization yielding few indications on transcription and expression pattern. For example, phage Sfi21, the prototype temperate *cos*-site *S. thermophilus* phage, has a 40-kb genome that potentially encodes 53 genes (Lucchini *et al.,* 1999b). Database searches allowed attribution of a likely function to only 10 genes. For dairy phages this is a fairly typical case. Phage O1205, the prototype temperate *pac*-site *S. thermophilus* phage with a similar genome size, but a totally unrelated structural gene cluster, also showed only 10 genes with good matches to entries from the database (Stanley *et al.,* 1997). It is currently unknown if this observation reflects an intrinsically great variability of phage genes or simply a limitation of the database. At present, only about 100 complete phage genomes are deposited in the database corresponding to less than two *Escherichia coli* genome equivalents.

Alternative approaches are therefore urgently needed to further our understanding of the genome organization in phages that are investigated for applied and not academic purposes (e.g., phage resistance in dairy microbiology, phage therapy in medical microbiology, and phage ecology in marine microbiology). One successful approach was the comparative genomic analysis of *S. thermophilus* phages that differed in phenotype (host range; susceptibility to phage resistance mechanisms; life-style: temperate vs virulent; DNA packaging mechanism: *cos*-site vs *pac*-site) (Lucchini *et al.,* 1999a,b). These comparisons allowed a given phage phenotype to be assigned to single gene (putative antireceptor protein) or larger DNA segments (putative lysogeny, DNA packaging, DNA replication modules, and structural

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gene clusters). Another approach hinged on the observation that *S. thermophilus* phages closely matched the gene map of lambdoid coliphages over the late gene region (Desiere *et al.,* 1999). An evolutionary analysis suggested that this similarity reflected ancient phylogenetic relationships between both phage groups (Brüssow and Desiere, 2001). On the basis of the genome map alignments with lambdoid phages (Casjens *et al.,* 1992), a number of gene functions have been predicted. In fact, the identity of the major head and major tail proteins were confirmed by electron microscopy combined with N-terminal sequencing (Desiere *et al.,* 1999; Stanley *et al.,* 1997). In addition, the tail tape protein (Pedersen *et al.,* 2000), the tail absorption protein (Duplessis and Moineau, 2001), the holin, and the lysin genes (Sheehan *et al.,* 1999) have been defined in dairy phages by a combination of genetic and biological experiments.

In the present article we report a transcription map for the temperate *cos*-site *S. thermophilus* phage Sfi21 by using 34 different DNA probes and confirming all deduced transcript locations by primer-extension experiments. The transcription map was used to crosscheck the *in silico* predictions of comparative phage genomics. The transcription map also provided a first insight into the dynamics of gene expression of phage Sfi21. In contrast to temperate lactococcal phages (Madsen and Hammer, 1998) the transcription of the temperate phage Sfi21 during the lytic growth was not initiated in the genetic switch region predicted by *in silico* analysis. The divergently transcribed genes of the postulated genetic switch region gave rise to middle transcripts. Chloramphenicol-inhibition studies suggested that the prominent lytic transcript of this region was under positive control by a phage protein encoded in early genes.

RESULTS

Lytic growth of the temperate *S. thermophilus* phage Sfi21

S. thermophilus cell strain Sfil was infected with the temperate phage Sfi21 at an OD (600_{nm}) of 0.1 with a multiplicity of infection of 10. Intracellular progeny phage was detected at 30 min postinfection (p.i.) and reached maximal titers at 50–60 min p.i., when the bacterial culture underwent lysis. Extracellular phage titers followed the intracellular phage titers with a 10- to 20-min time lag (data not shown). Until shortly before cell lysis, infected and uninfected cells could not be distinguished by their polypeptide pattern on SDS–PAGE (data not shown). Cells infected with the temperate phage Sfi21, but not those infected with a virulent *S. thermophilus* phage, resumed growth in milk with a time lag of about 4 h. All survivors contained the Sfi21 prophage. Based on a doubling time of 25 min for *S. thermophilus* cells, we can estimate that approximately 1 of 1000 infected cells took the lysogenic instead of the lytic pathway. Under single-step growth conditions a comparable growth curve was obtained for phage Sfi21 (data not shown). The burst size was about 150 phage particles per cell. Comparison of the viable cell counts and infective centers demonstrated again that about 1 in 1000 cells survived the infection.

Transcription mapping in phage Sfi21

Total RNA was isolated from the infected cells at 0 and 2 min p.i., and then every 5 min until about 40 min p.i. Fifteen micrograms of total RNA were separated on a denaturing agarose gel, blotted, and phage transcripts were revealed by Northern blot hybridization using specific DNA probes. The precision of the localization of the phage mRNAs on the phage genome map is limited by the number of DNA probes used in Northern blots and their spatial resolution. More than 30 DNA probes were used to localize the transcripts on the genome map. To increase that resolution, the 5′ ends of all transcripts were mapped by primer-extension experiments. The 3′ position of the mRNA was deduced from the estimated size of the mRNAs and from hybridization experiments with probes corresponding to adjacent DNA segments. Rho-independent terminators were predicted by *in silico* analysis. A summary of the results is presented in the transcription map shown in Fig. 1.

Transcription of the predicted genetic switch region

DNA sequence analysis of phage Sfi21 predicted a genetic switch region between ORF 127 and 75, two divergently transcribed genes that encode repressor-like proteins (Fig. 1) (Bruttin *et al.,* 1997b). ORF 127 and the following three genes represent the only leftward transcribed gene clusters of the phage Sfi21 genome and included a biologically proven superinfection immunity gene (ORF 203) (Bruttin *et al.,* 1997b) and the phage integrase (ORF 359) (Bruttin *et al.,* 1997a) (Fig. 1). When the Northern blots were probed with ORF 127, the predicted *cI*-like repressor gene, only weak phage transcripts were detected (Fig. 2C) and no primer extension products were obtained with ORF127-specific primers from RNA of lytically infected cells (data not shown). A 1.6- and a 3.2 kb-long mRNA species was detected with the ORF 127 probe only after longer exposure times (Fig. 2C′). When Northern blots were probed with ORF 75, the predicted *cro*-like repressor gene, a single 2.1-kb hybridization signal was detected by autoradiography (Fig. 2A). The signal was clearly visible at 12 min p.i., increased in intensity until 27 min p.i., and decreased at later times of infection. The maintenance of a signal until late in infection could indicate ongoing transcription or stability of the mRNA synthesized at an earlier time of infection. Primer-extension experiments located the 5′ end 45 bp upstream of the start codon of ORF 75 (Fig. 2B). The ORF 75 start codon is preceded by a good ribosomal binding site (RBS) and the 5′ end of the transcript is preceded by

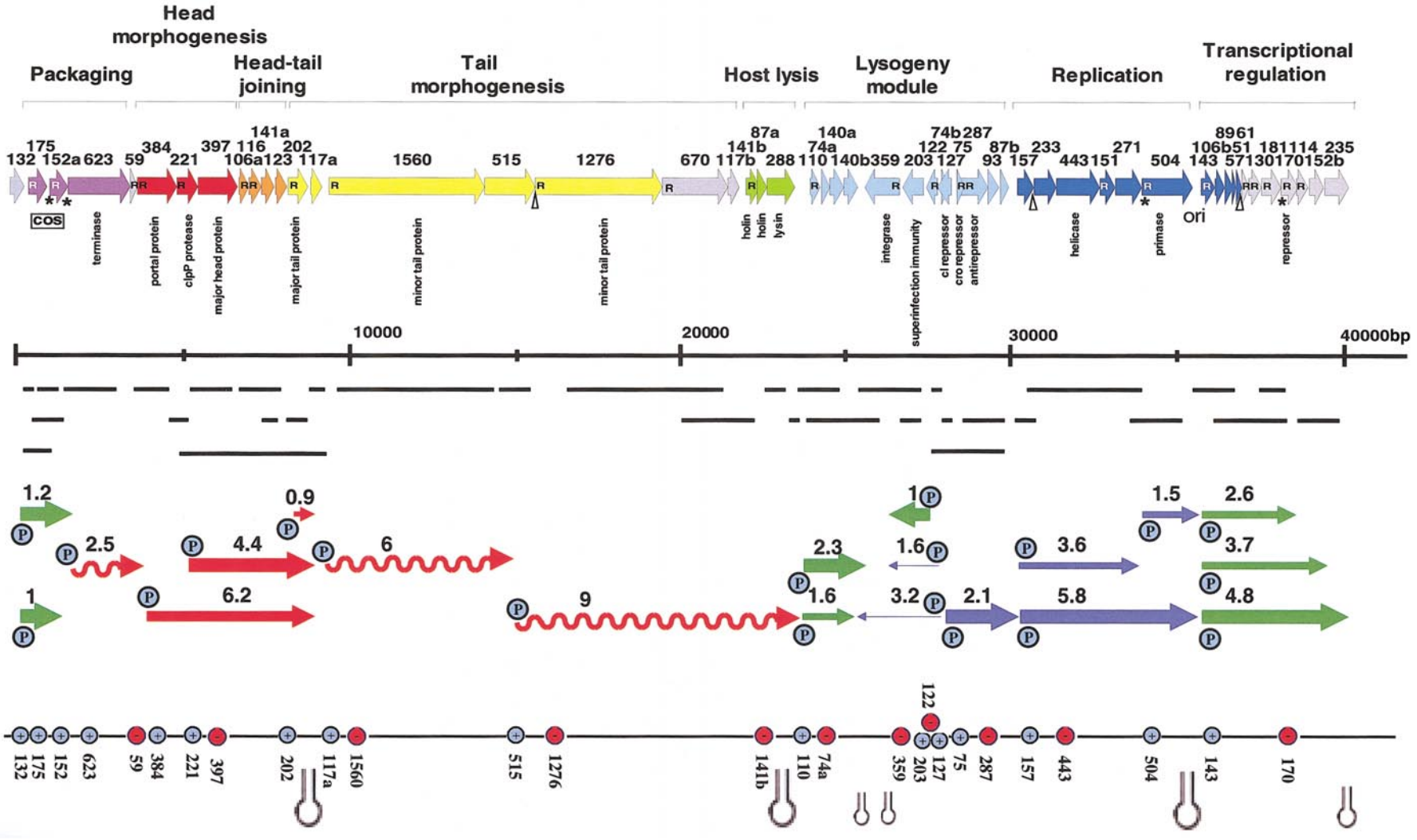

FIG. 1. Transcription map for the temperate *S. thermophilus* phage Sfi21. (Top) Prediction of the open reading frames in the complete genome of phage Sfi21. The ORFs are marked with their length in codon numbers. Probable gene functions identified by bioinformatic analysis are noted below the map. Gene functions identified by biological experiments are underlined. The phage genome was divided into functional units as predicted by previous bioinformatic and comparative evolutionary analysis (Lucchini *et al.,* 1999b). The modules are indicated by brackets at the top of the gene map. Genes predicted to belong to the same unit have the same color. Gray filling indicates lack of information about the probable function of the indicated ORFs by *in silico* analysis. ORFs preceded by a potential RBS are marked with an R inside the arrow. ORFs starting with an unconventional initiation codon are indicated with an asterisk. Overlap of start and stop codon is indicated with a triangle. (Middle) The approximate position of the PCR products used for probing of the Northern blots is provided with the scale in base pairs. (Bottom) Summary of the transcription analyses. The Sfi21-specific transcripts are depicted as arrows; the arrows point to the 3′ end of the mRNA. The arrows are colored in green, blue, or red to indicate early, middle, and late transcripts, respectively. The length of the arrow is proportional to the length of the mRNA derived from the Northern blots. The estimated size of the mRNA is indicated in kilobase pairs. The transcripts are positioned with respect to the genome map shown above. The width of the arrows indicates the relative abundance of the mRNA species. The wavy lines indicate mRNAs that presented as smeared hybridization results. Positive (blue circles) and negative (red circles) results of the primer-extension experiments are noted next to the identification of the tested ORF. Hairpins indicate possible rho-independent terminators; the two sizes of the hairpins refer to different energies calculated for the hairpin (large: >15 kcal/mol; small: 10–15 kcal/mol).

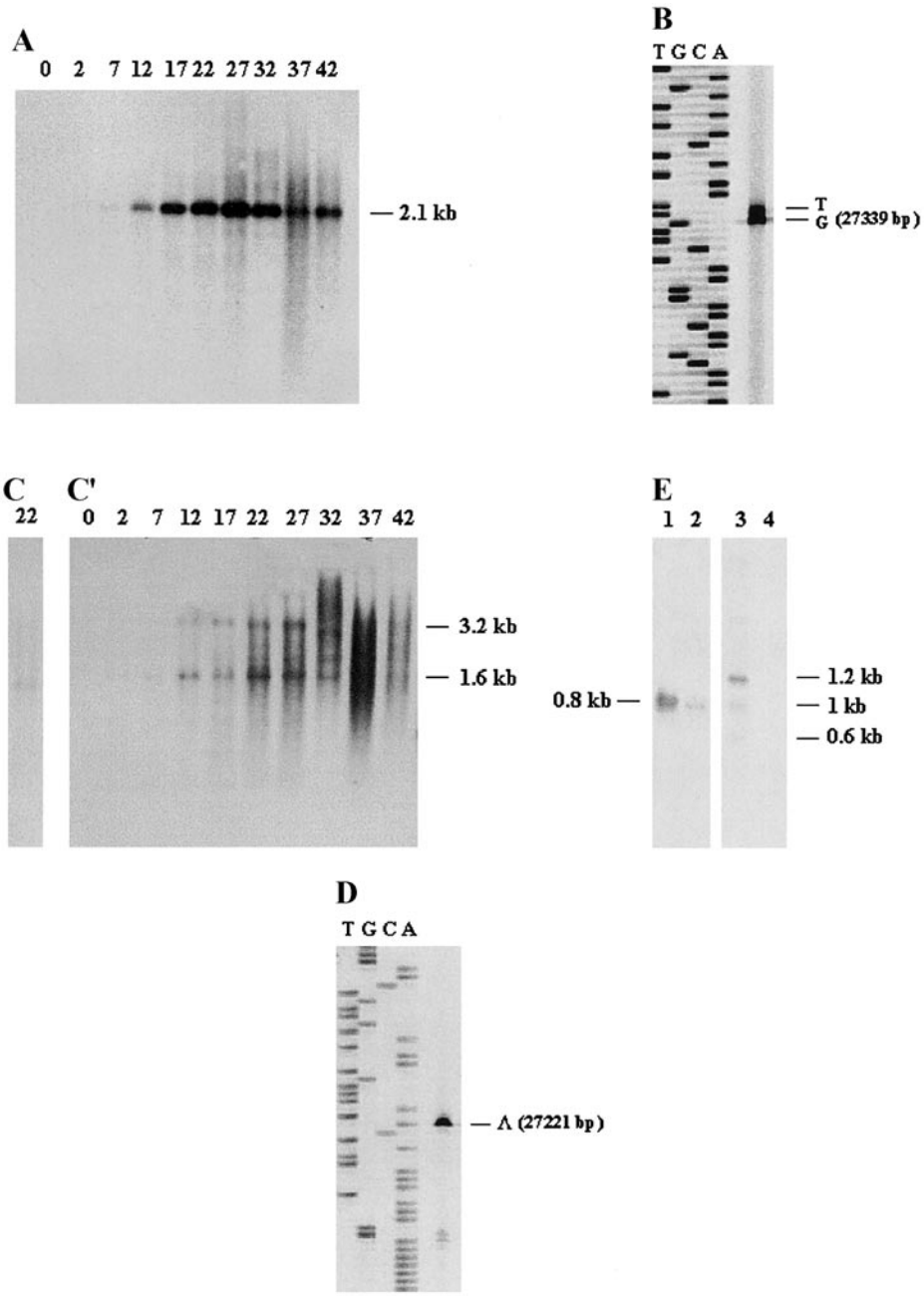

FIG. 2. Northern blot of total RNA isolated from *S. thermophilus* SfiI at the indicated time after infection (0–42 min p.i., indicated at the top of the Northern blots) with phage Sfi21 were hybridized against an ORF75-specific DNA (A) or an ORF127-specific DNA (C, C′ shows a six-fold longer exposure of the autoradiography compared to A). The estimated size of the detected transcripts in kb are indicated to the right of each panel. Primer-extension analysis carried out with transcripts from Sfi21-infected cells with oligonucleotides placed near the 5′ ends of the transcripts starting from ORF 75 (B) and from ORF 127 (D, RNA from SfiI cell transformed with plasmid pSFcI). The major primer-extended products are identified with respect to entry NC_000872. E: ORF127-specific (lanes 1, 2) and ORF 75-specific (lanes 3, 4) mRNA expression in SfiI cells containing plasmids pSFcI (lane 1), pSFswi (lanes 2, 4), and pSFcro (lane 3). Molecular weights are indicated in kb.

nearly perfect −10 and −35 consensus promoter sites for *L. lactis*, a close evolutionary relative of *S. thermophilus*.

Transcription of the cloned genetic switch region

When ORF 127 was cloned on plasmid pNZ124 under the control of the intergenic region between ORF 127 and 75, an ORF 127-specific messenger was detected in transformed *Sfi*I cells (Fig. 2E, lane 1) and the putative transcription start site could be determined by primer extension (Fig. 2D). The 5′ end suggested the use of an internal in-frame start codon in ORF 127. Neither a good RBS nor a consensus promoter structure, respectively, preceded the predicted start codon and the 5′ end of the

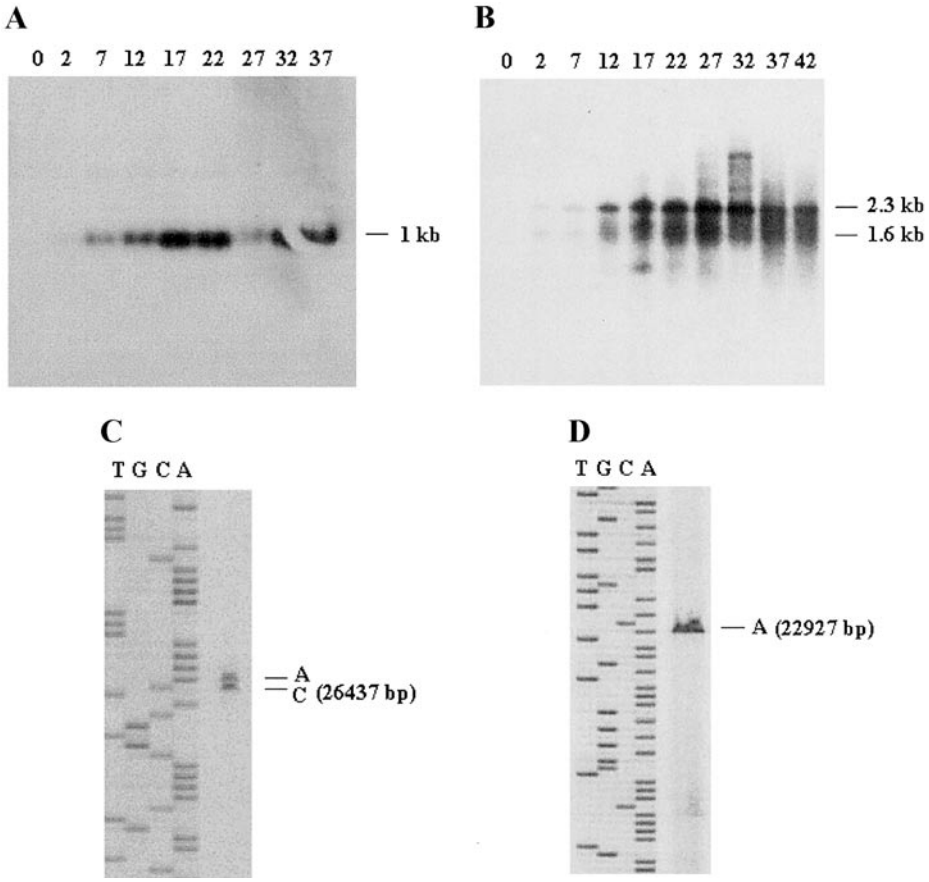

FIG. 3. Northern blot and primer extension analysis of early transcripts in the lysogeny module. Northern blots of total RNA isolated from Sfi21-infected cells at the indicated times p.i. were hybridized against an ORF 203-specific (A) and an ORF 110 to140b-specific probe (B). Major transcripts were identified with their length in kb. Primer extension analysis of the transcripts starting at 5′ end of ORF 203 (C) and 5′ end of ORF 110 (D). The major primer-extended products are identified with respect to entry NC_000872.

transcript. An ORF 127-specific mRNA of identical size was also observed (Fig. 2E, lane 2) when ORF 127 was cloned in the context of the putative genetic switch region comprising ORF 127 to 93, thus including the putative *cro*- and *ant*-like repressor genes (see Fig. 1 for orientation). An ORF 75-specific mRNA was seen when ORF 75 was cloned on plasmid pNZ124 under the control of the intergenic region (Fig. 2E, lane 3). The 5′ end determined in primer-extension experiments corresponded to that seen in lytic infection (data not shown), but transcription apparently continued into the plasmid DNA and was terminated at several nonspecific sites. In contrast, no ORF 75-specific mRNA was detected in Sfil cells transformed with the entire putative genetic switch region comprising ORF 127 to 93 (Fig. 2E, lane 4). This suggests negative regulation by the repressor encoded in ORF 127.

Transcription of the remaining lysogeny genes

A probe covering the superinfection immunity gene (ORF 203) detected a 1-kb-long mRNA species. This mRNA became detectable at 7 min p.i. and reached maximal transcription levels at 17 min p.i. (Fig. 3A). Primer-extension experiments placed the 5′ end of the transcript 48 bp upstream of the predicted start codon of ORF 203 (Fig. 3C).

A group of genes located between the lysin and the integrase genes of phage Sfi21 have been tentatively assigned to the lysogeny module (Lucchini *et al.,* 1999b). These genes were transcribed during lytic infection and presented as a major 2.3-kb-long mRNA and as less well-defined further transcripts. The transcription started early and the transcripts were maintained throughout the entire infection cycle (Fig. 3B). Primer-extension analysis (Fig. 3D) placed the 5′ end 25 bp upstream of ORF 110 and was preceded by a consensus promoter.

Transcription of the DNA replication module

A comparative genomics analysis identified a highly conserved DNA segment in *S. thermophilus* phages covering ORF 157 to 51 (Desiere *et al.,* 1997). The predicted proteins showed similarity with helicases, primases, and nucleoside triphosphate (NTP)-binding proteins, respectively. An origin of phage replication was demonstrated

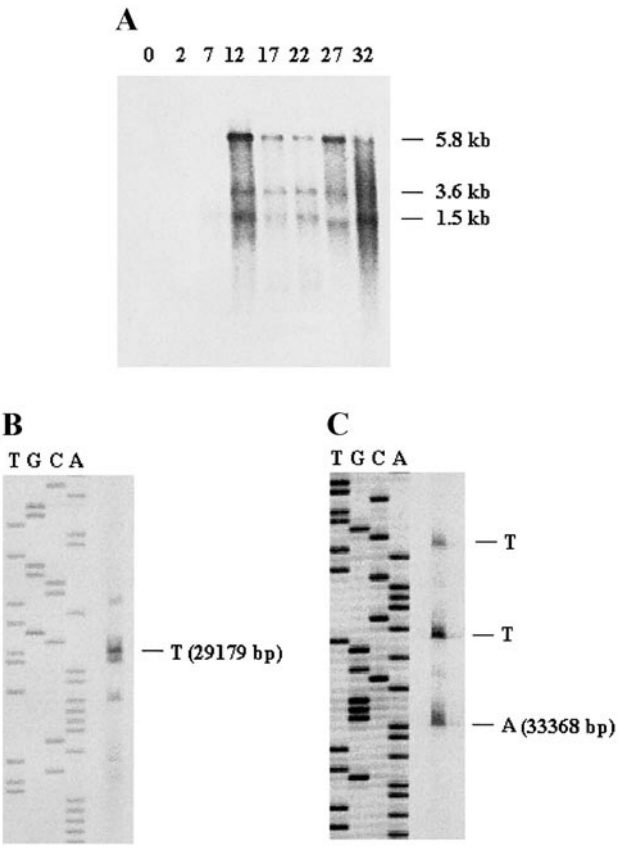

FIG. 4. Northern blot and primer extension analysis of middle transcripts. (A) Northern blot hybridized with the ORF 443-specific DNA probe from the DNA replication module. The three major transcripts are identified with their sizes in kb. Primer extension analysis of the 5′ ends of RNA transcripts from ORF 157 (B) and from ORF 504 (C). The major primer-extended products are identified with respect to entry NC_000872.

experimentally in phage Sfi21 and located downstream of ORF 504 (Foley *et al.,* 1998). Virulent *S. thermophilus* phages showed a second origin of replication downstream of ORF 51 (Lucchini *et al.,* 1999b), leading to the prediction of the DNA replication module as depicted in Fig. 1. A probe covering ORF 443 (helicase) to ORF 504 (primase) revealed three mRNA species of 5.8, 3.6, and 1.5 kb (Fig. 4A). These phage mRNAs were detected from 12 min p.i. until the end of the infection cycle. A probe covering ORF 157 hybridized with the 5.8- and 3.6-kb mRNA species, while a probe covering ORF 504 hybridized with the 5.8- and 1.5-kb transcript (data not shown). Primer extension analysis located the 5′ ends of a transcript 75 bp upstream of ORF 157 (Fig. 4B) and three 5′ ends of phage transcripts were placed upstream of ORF 504 (Fig. 4C).

From ori to *cos* site

In contrast to previous *in silico* predictions, the genes downstream of the primase gene were found to belong to a distinct transcription unit (Fig. 1). Both a probe consisting of ORF 143 to 51 and a probe comprising ORF 181 to 170 revealed a 4.8-kb early mRNA as the major transcription product and less well-defined mRNA species in the size range of 3.7 to 2.6 kb (Fig. 5A, data not shown). Primer extension located the 5′ end of the major early phage transcript 94 bp upstream of ORF 143 (Fig. 5B).

A probe corresponding to ORF 132 identified two further early phage transcripts with 1.2- and 1-kb molecular weight (Fig. 5C). The 1.2-kb transcript was detected first; it was gradually replaced by the 1-kb transcript from 12 min p.i. and both transcripts were drastically reduced at 27 min p.i. The 5′ end of the transcript was located 46 bp upstream of ORF132 (Fig. 5D).

Transcription of DNA packaging genes

Comparative genomics supported the identification of three genes that flanked the *cos*-site (ORF 175, 152a, and 623) as likely DNA packaging genes (Lucchini *et al.,* 1999b) (Fig. 1). ORF 623 shared sequence similarity with large subunit terminases (Desiere *et al.,* 2001a). Primer-extension experiments demonstrated three 5′ ends for transcripts of this region: the first site is 77 bp upstream of ORF 175 (Fig. 6A); the second site is 89 bp upstream of ORF 152 (which can only be transcribed when the *cos*-site is sealed) (Fig. 6B), and the third site is 70 bp upstream of ORF 623 (Fig. 6C). However, several different RNA preparations failed to yield distinct hybridization signals when using various DNA probes from this region. A smear of signals was observed starting from 17 min p.i. to the end of the infection cycle. The peak distribution of the smear was between 2.5 and 1 kb (data not shown).

Transcription of structural genes

Two mRNA species of 6.2 and 4.4 kb size (Fig. 7) covered the genome region that was associated with head morphogenesis (Desiere *et al.,* 1999) (see Fig. 1 for orientation). A weak signal was seen at 17 min p.i. and transcripts became prominent between 22 and 32 min p.i. A probe from the likely portal protein gene (ORF 384) revealed a defined 6.2 mRNA species plus some smeared RNA (Fig. 7A, the "band" at the lower edge of the smear is an artifact due to 23 S rRNA; see also Fig. 7B). In contrast, all probes corresponding to a protease gene (ORF 221) or ORF 106 to 123 (predicted to encode head-to-tail joining genes) or the major tail gene (ORF 202) yielded both the 6.2- and the 4.4-kb hybridization signal (Fig. 7B and data not shown). The ORF 202 probe also detected a small 0.9-kb transcript (data not shown). Primer-extension experiments located the 5′ ends of transcripts 20 bp upstream of the start codon from ORF 384 (portal protein, Fig. 7C), 21 and 230 bp, respectively, ahead of the start codon from ORF 221 (ClpP protease, Figs. 7D and D′), and 52 bp ahead of ORF 202 (tail gene)

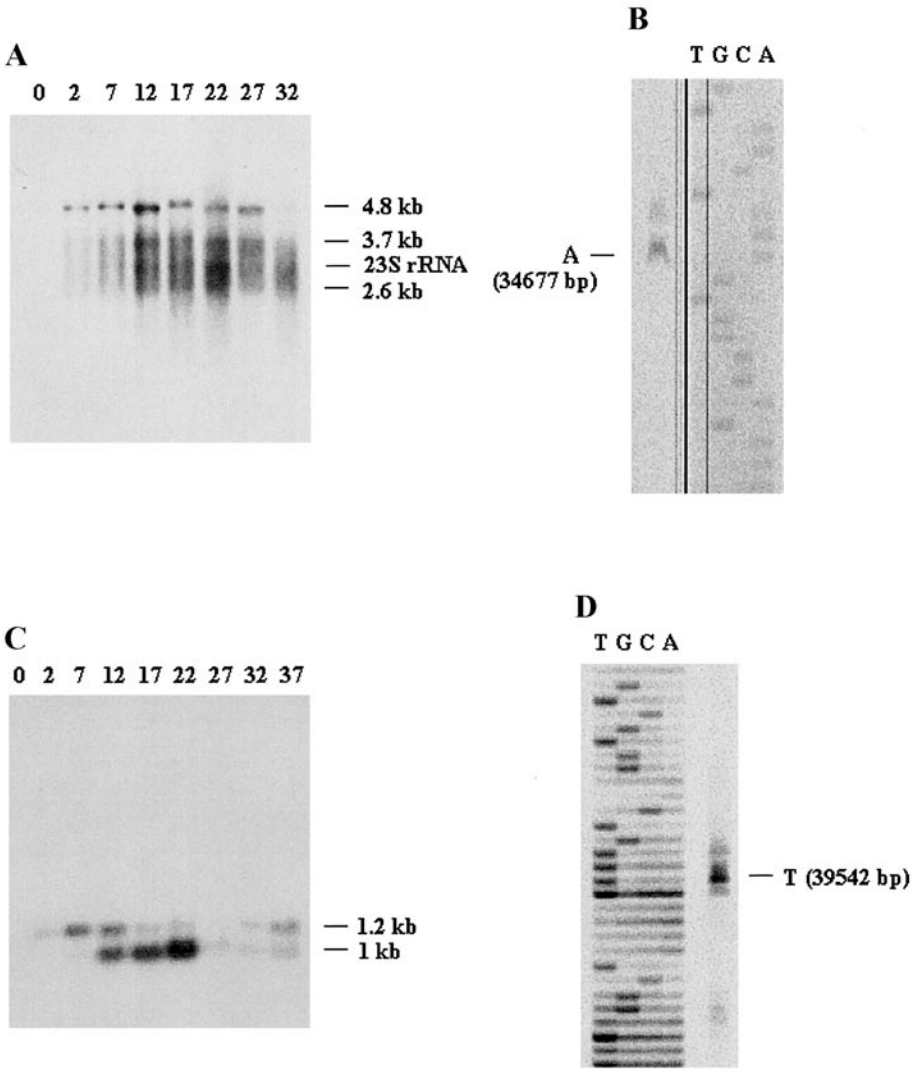

FIG. 5. Northern blot and primer extension analysis of the early transcripts located between DNA replication genes and the *cos*-site. (Left) Northern blots hybridized with a probe covering ORF143 to ORF 51 (A) and ORF 132 (C). (Right) Primer extensions analysis was done with oligonucleotides targeted to the 5′ ends from ORF 143 (B) and ORF 132 (D). The major primer-extended products are identified with respect to entry NC_000872.

(Fig. 7E). A rho-independent terminator was identified downstream of ORF 202.

Probes covering separately ORF 1560 (the putative tail tape measure gene), ORF 515, ORF 1276 (the putative tail absorption gene), ORF 670, or ORF 288 (the lysin gene) all revealed a smear of transcripts with the highest molecular weights at 6 kb (ORF 1560, ORF 515) or 9 kb (all other probes), respectively (Fig. 8A and data not shown). Maximal RNA accumulation was observed at 22–32 min p.i. and 5′ ends of phage transcripts over this region were located 28 and 70 bp upstream of ORF 117a and 515, respectively (Figs. 8B and 8C). Notably, no 5′ end was located upstream of the three genes constituting the lysis cassette (two holins followed by a lysin gene), suggesting cotranscription with tail fiber genes. A strong rho-independent terminator was identified downstream of ORF 288 (lysin).

Lack of a consensus promoter sequence ahead of the experimentally determined 5′-mRNA ends

If the 5′ ends of the phage transcripts are not processed, the 5′ ends of the phage mRNAs determined in primer extension experiments correspond to the transcriptional start sites and allow the identification of phage promoters by reference to the phage genome sequence. However, inspection of the upstream sequences from most phage Sfi21 transcripts did not reveal likely promoter sequences. Also an alignment of these upstream sequences yielded only a poor consensus sequence. For the eight early and middle transcripts of

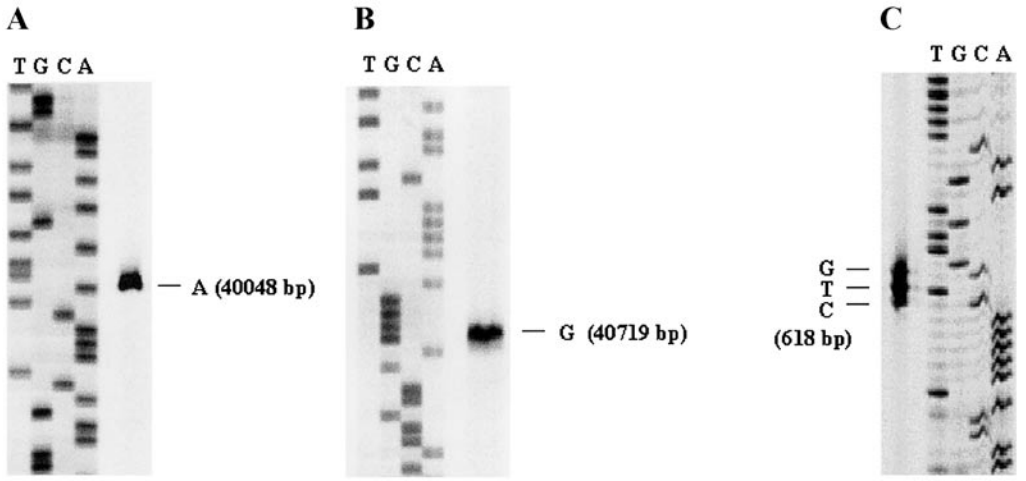

FIG. 6. Primer-extension experiments with the mRNAs from the putative DNA packaging module. The oligonucleotides were targeted to the 5′ ends from ORF 175 (A), ORF 152 (B), and ORF 623 (C). The major primer-extended products are identified with respect to entry NC_000872.

phage Sfi21, the following consensus was derived: AATxxAaAxAxaxxtxxAxaxaxatGTtaTaaaxAAaxt-first base pair of primer extension product. A potential extended −10 promoter site is underlined. Uppercase indicates conservation in more than or equal to five of the eight transcripts, and lowercase indicates conservation in four

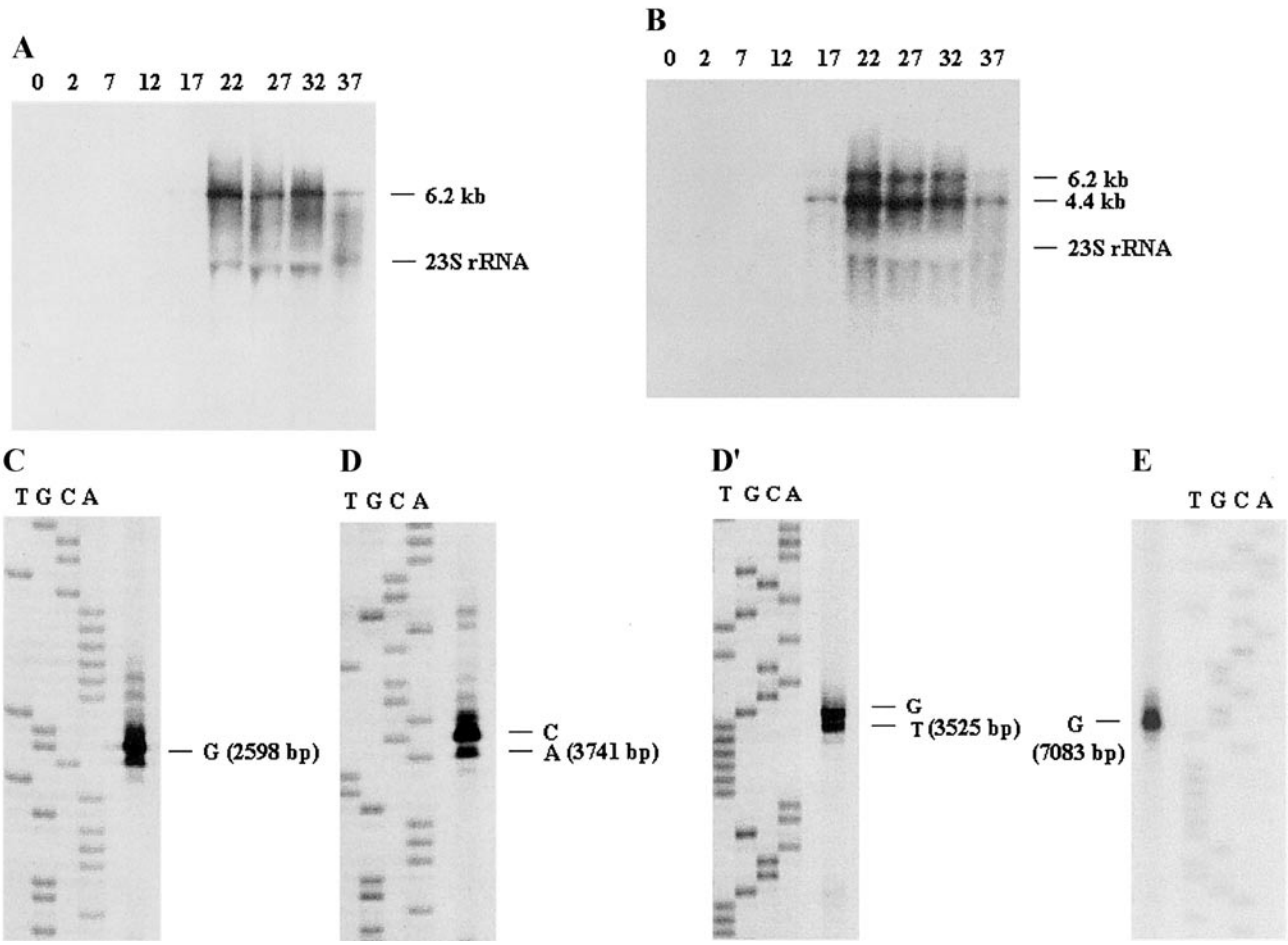

FIG. 7. Northern blot and primer extension analysis of late transcripts located in the head morphogenesis module. (Top) Northern blot analysis using probes corresponding to ORF 384 (A) and to a region covering ORF 106 to ORF123 (B). (Bottom) Primer extensions analysis was done with oligonucleotides targeted to the 5′ ends from ORF 384 (C), ORF 221 (D, D′), and ORF 202 (E). The major primer-extended products are identified with respect to entry NC_000872.

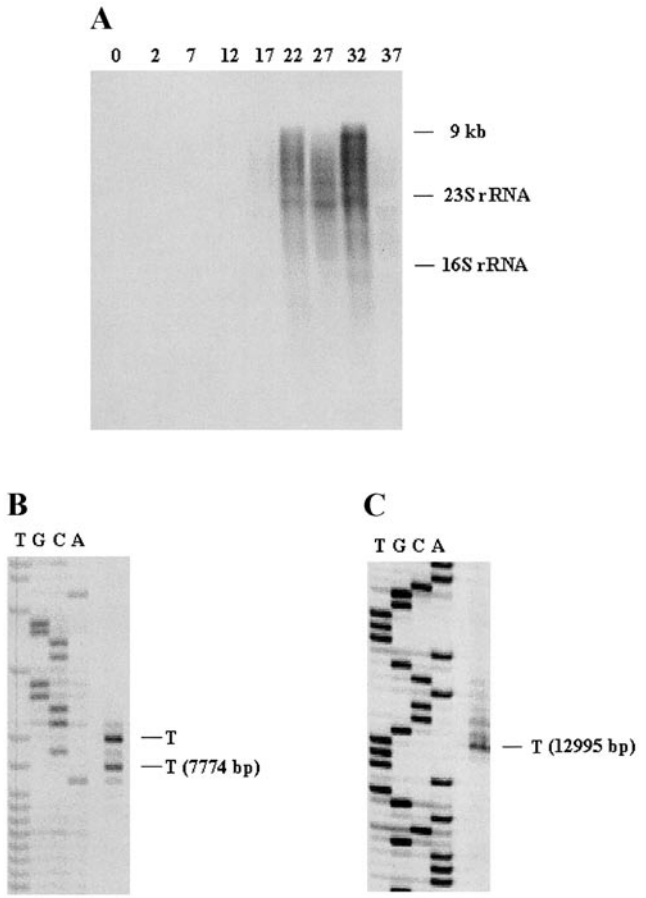

FIG. 8. Northern blot and primer extension analysis of late transcripts located in the tail morphogenesis module. (Top) Northern blot hybridized with a probe covering ORF 670 to 288 (A). (Bottom) Primer extensions analysis was done with oligonucleotides targeted to the 5′ ends from ORF 117a (B) and ORF 515 (C). The major primer-extended products are identified with respect to entry NC_000872.

transcripts. X indicates nonconserved sites. No conserved −35 promoter site was detected. Even less conservation was seen for the upstream sequences of the late transcripts (data not shown). In contrast, 10 of 16 putative mRNAs showed a ribosomal binding site appropriately spaced with respect to the predicted start codon.

The lack of a consensus promoter sequence upstream of the phage transcripts could suggest that the synthesis of a phage-encoded product is needed to initiate transcription. To address this possibility, protein synthesis was blocked by the addition of chloramphenicol at the time of phage infection. The effect of 10, 20, and 30 μg chloramphenicol per milliliter on early-, middle-, and late-expressed phage transcripts was tested. Measures of 20 and 30 μg of chloramphenicol per milliliter suppressed all phage transcription (data not shown). In contrast, addition of 10 μg/ml chloramphenicol suppressed middle- and late-phage transcripts only (Figs. 9A–9D). Primer-extension experiments confirmed these results (Figs. 9E–9G). We were especially interested in the effect of chloramphenicol on the transcription from the genetic switch region. The transcription level of ORF 127 repressor was as low in chloramphenicol-treated as in control cells (Fig. 10B). Interestingly, the 2.1-kb mRNA detected with an ORF 75 repressor-specific probe in control cells was not observed in chloramphenicol-treated cells (Fig. 10A).

DISCUSSION

Transcription mapping offers a "reality check" for *in silico* predictions in phage genomics. Since it also reveals dynamic aspects of the phage genome expression, it can provide insights into the phage genome organization that cannot be readily predicted by DNA sequence analysis. Transcription mapping is thus a useful second step after phage DNA sequencing and before biochemical or genetic experiments are done. However, while genomic analysis can be performed with any concentrated phage preparation, transcription mapping depends on the isolation of phage plaques and the *in vitro* cultivation of the bacterial host.

What are the lessons from transcription mapping for the understanding of the genome organization in *S. thermophilus* phage Sfi21?

Lysogeny module

Comparative genomics of temperate and virulent *S. thermophilus* phages led to the proposal of a lysogeny module, which included a putative genetic switch region. Transcription mapping confirmed the right border of this *in silico* prediction, while it slightly corrected the left border of the lysogeny module by one gene (ORF 110).

For temperate phages of dairy bacteria a transcription map has only been reported for the *L. lactis* phage TP901-1 (Madsen *et al.,* 1998). Its transcription map differs from that of Sfi21 in an important aspect: All early transcripts in TP901-1 were initiated in the predicted genetic switch region. A similar transcription pattern was observed for another *pac*-site temperate lactococcal phage, Tuc2009 (D. van Sinderen, personal communication). In phage Sfi21, however, the divergently oriented promoters of the putative genetic switch region are transcriptionally silent in the early infection phase. The transcriptional silence of both repressor genes during early infection is surprising since ORF 75 has a consensus promoter and ORF 127 is transcribed from its own promoter when cloned on a plasmid. This indicates that both genes can be transcribed by an unmodified host RNA polymerase.

Early genes

Transcription mapping located early genes in the lysogeny module and between the DNA replication module and the *cos*-site. The latter region gave rise to two major transcripts, one 4.8-kb-long mRNA extending over

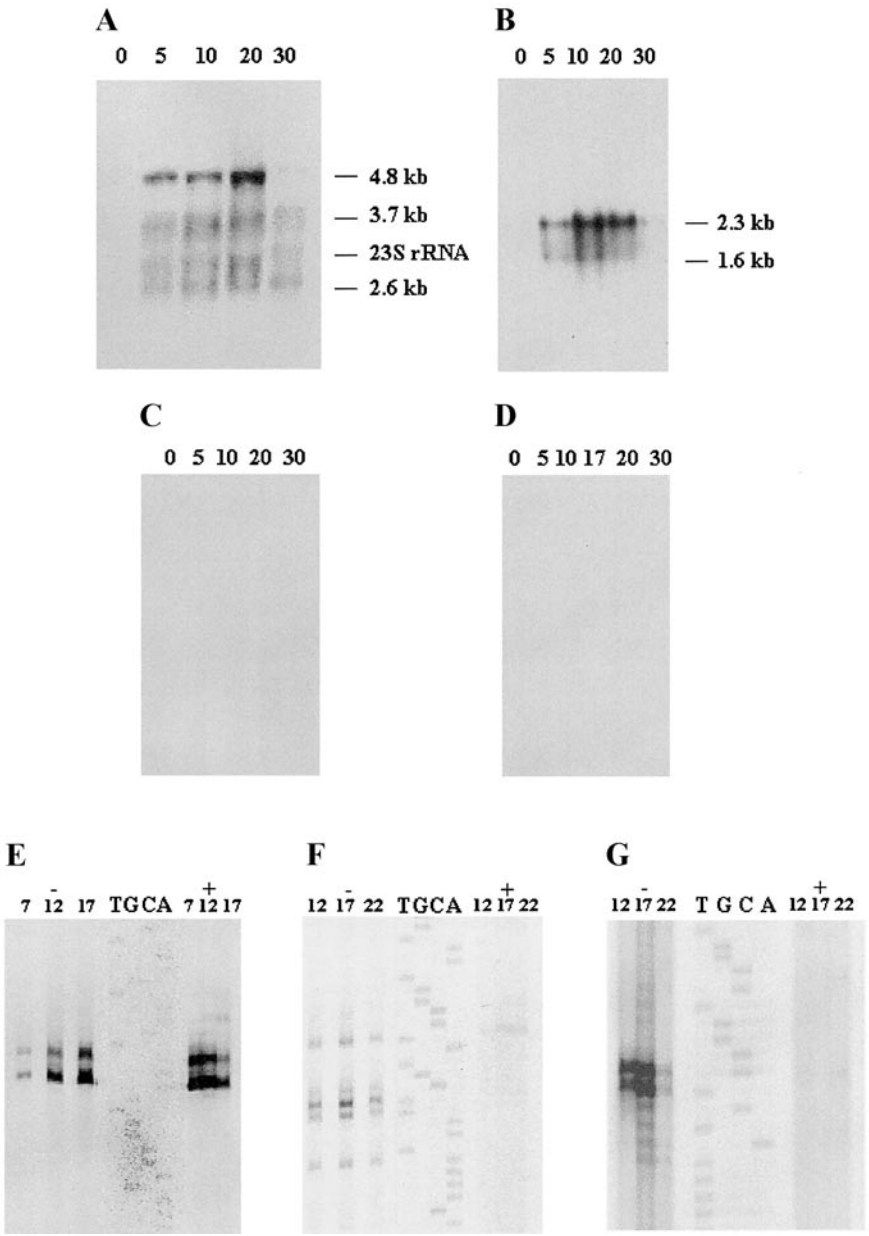

FIG. 9. Early, but not middle, and late transcripts are made in the presence of 10 μg/ml of the protein synthesis inhibitor chloramphenicol. (Top) Northern blots of mRNA isolated at the indicated times after infection of chloramphenicol-treated cultures were hybridized against the following probes. (A) Early genes ORF 181 to 170; (B) early genes ORF 110 to 140b; (C) middle gene 157 (DNA replication module); and (D) late gene 384 (portal protein gene). (Bottom) Primer extensions analysis was done with oligonucleotides targeted to the 5′ ends from early gene ORF 143 (E), middle gene ORF 157 (F), and late gene ORF 117a (G). Three primer extension experiments are shown for RNA preparations recovered at 7, 12, and 17 min p.i. (E) and at 12, 17, and 22 min p.i. (F and G) from control (−) and chloramphenicol (+)-treated cultures.

12 genes and another mRNA covering a single gene (ORF 132). Comparative genomics has correctly set apart ORF 132 from the rest of the genome. The right border of the module defined by the 4.8-kb mRNA was also correctly predicted (Lucchini *et al.,* 1999b). However, the left half of the 4.8-kb mRNA was erroneously assigned to the DNA replication module based on the observation of a duplicated origin of replication in the corresponding genome region of virulent *S. thermophilus* phages. ORF 143 to 51 are part of the 4.8-kb early transcript, which makes the *in silico* prediction as DNA replication genes untenable. The left border of the DNA replication module was correctly predicted by *in silico* analysis (Desiere *et al.,* 1997). The newly defined right border of the DNA replication module ends now with an experimentally proven origin of replication (Foley *et al.,* 1998).

The transcription of middle and late genes, but not of early genes, was suppressed when the cells were infected in the presence of a protein synthesis inhibitor. This data suggest that the early Sfi21 genes encode a

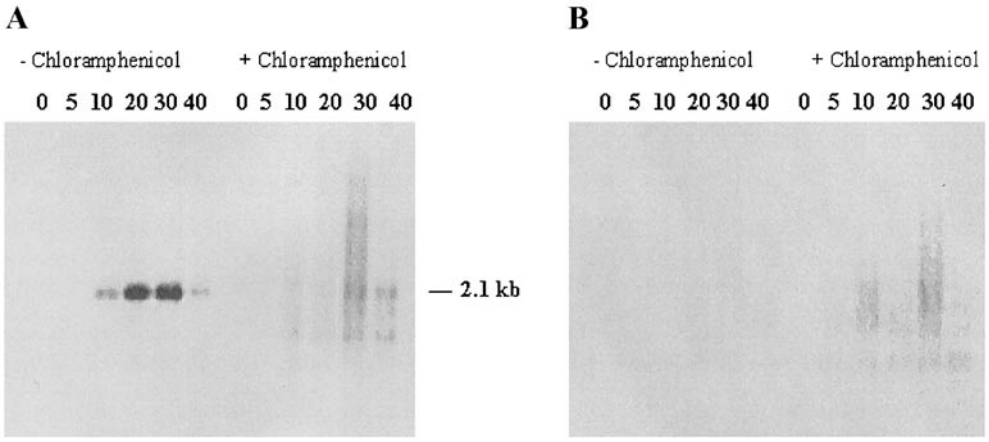

FIG. 10. Effect of chloramphenicol on the transcription of the genetic switch region. (A) Northern blots of RNA isolated from phage-infected cultures at the indicated times p.i. The infections were done in the absence (−) or presence (+) of chloramphenicol. The blots were probed with ORF 75 repressor (A) or ORF 127 repressor (B).

transcription regulator for middle and late gene transcription. Notably, the transcription of the major lytic mRNA from the putative genetic switch region was also under the control of such a protein(s). This means that the genetic switch region is not the major regulatory site for the transcription of the Sfi21 genome at least under conditions of lytic growth. In fact, the expression of the 2.1-kb mRNA from the genetic switch region is not necessary for the progression of the lytic program. First, the transcription of the 2.1-kb mRNA did not precede the transcription of the DNA replication genes. Second, a number of deletion mutants were isolated from Sfi21 that had lost various parts of the genetic switch region including the *cro*-like gene without compromising the lytic growth capacity of the derivative phages (S. Foley *et al.,* unpublished observations). It is possible that the genetic switch region is dispensable for the lytic infection cycle of Sfi21 and only necessary during establishment and maintenance of the lysogenic state and the induction from the prophage state.

Only a single early gene, ORF 170, transcribed from the 4.8-kb early mRNA, provided informative database matches and is a good candidate for a transcription regulator. Over the N-terminal half it shared significant similarity with a possible transcription regulator from *Bacillus subtilis* and repressors from phages infecting a large range of bacteria. The similarity covered the helix-turn-helix DNA binding region of the coliphage 434 repressor. Over its C-terminal half it shared similarity with a probable regulatory protein from enterococci (Lucchini *et al.,* 1999a). In contrast to the situation in the virulent *L. lactis* phage c2 (Lubbers *et al.,* 1995), no like factor was identified on the Sfi21 genome. Also in contrast to phage c2, no clear transition from early consensus to late nonconsensus promoters was seen in phage Sfi21, suggesting a distinct type of transcription regulation in both phages.

Our primer-extension results deserve a comment. Primer extension determines the 5′ end of the transcripts as they are found within the cell; they do not necessarily correspond to the transcription initiation sites. In fact, few phage Sfi21 transcripts were preceded by standard −10 and −35 promoter sites when inspected visually. Several reasons could account for this observation. First, the 5′ ends of the phage Sfi21 transcripts might be processed and might not correspond to the authentic transcription initiation site. Since vaccinia virus guanylyltransferase can only add a radiolabeled cap to RNA having a di- or triphosphate at the 5′ end, only ends generated by initiation of transcription will be labeled (Hsu *et al.,* 1990). In preliminary experiments, RNA was isolated from infected cells during the late infection phase when Northern blots demonstrated the presence of most early, middle, and late transcripts. The RNA was radiolabeled with guanylyltransferase and the labeled RNA was hybridized against a slot blot DNA array of PCR products representing all segments of the phage genome. Interestingly, only tail fiber genes, but no DNA packaging, head or tail genes hybridized with the labeled late RNA probe (M. Ventura, unpublished results).

Second, several mRNA had multiple start sites. This creates problems for the alignment and the definition of consensus upstream sequences. In most cases the multiple 5′ ends were close to each other, which is not unusual for prokaryotic promoters. In a few cases, however, multiple 5′ ends were identified (ORF 157, 504, and especially 221, where two major putative start sites were spaced 200 bp apart). Third, the lack of sequence conservation of the upstream sequences might reflect a lesser degree of sequence conservation in *S. thermophilus* than in *L. lactis*. A compilation of cellular streptococcal genes (Constable and Mollet, 1994; Sabelnikov *et al.,* 1995; Stingele and Mollet, 1996; Vaughan *et al.,* 2001) also showed no clear consensus promoter sequence

(data not shown). Fourth, the chloramphenicol experiments suggest that a phage protein(s) encoded by an early gene is necessary for the transcription of middle and late genes, suggesting nonconsensus promoters. To identify the phage Sfi21 promoter sequences, the upstream sequences must be directly tested for promoter activity in promoterless indicator vectors. Two technical problems make this approach not straightforward. Common promoter screening vectors from *L. lactis* cannot be used in *S. thermophilus*, which has for example a much higher endogenous β-galactosidase and β-glucuronidase activity than *L. lactis*. In addition, the chloramphenicol experiments suggest that most of the identified promoters will not be active in the absence of supplementary early phage factor(s).

Late genes

The *in silico* definition of the DNA packaging module was confirmed by transcription analysis. The packaging region gave rise to defined late transcripts, whereas transcripts of the tail morphogenesis and the lysis modules gave a smear of mRNAs. Degradation of late transcripts by nonspecific cellular RNases is unlikely since late transcripts from the head morphogenesis module showed a distinct hybridization signal until late in the infection cycle. The smearing of late transcripts has been observed in a number of lactococcal phages (sk1, Tuc2009, bIL41; Chandry *et al.,* 1994; Parreira *et al.,* 1996; van Sinderen, personal communication). Segmental differences in the stability of polycistronic mRNAs have also been reported for a number of phage operons (Mudd *et al.,* 1990). In analogy to the T4 phage system, Parreira *et al.* (1996) proposed RNase E cleavage for late transcripts from lactococcal phages. Putative pentanucleotide consensus sequences (A/G)AUU(A/U) for RNase E recognition were detected in a number of late ORFs from phage Sfi21 (ORF 623, 117a, 1276, 288). However, the predictive value of such consensus sites has been questioned (Cohen and McDowall, 1997). It is therefore unclear whether specific RNase can explain the instability of selected late transcripts.

The mRNA smearing prevented the definition of the border between tail fiber genes (last clear representative: tail absorption gene/ORF 1276) and the lysis module (first clear representative: holin/ORF 141b). Primer-extension experiments suggested that the lysis genes are cotranscribed with the preceding tail fiber genes. The next possible transcript putative start sites were placed ahead of ORF 670 (weak signal, data not shown) and ORF 515. The control of the phage Sfi21 lysin activity probably occurs at the protein level. Possibilities for lysin control are provided by the presence of two holin genes in *S. thermophilus* phages (Sheehan *et al.,* 1999) and the dual start motif (Bläsi and Young, 1996; Bruttin *et al.,* 1997a) in holin ORF 87a gp.

Interestingly, the 6.2-kb late transcript covered genes from three distinct modules (head morphogenesis, putative head-to-tail joining genes, and major tail protein). Cotranscription of the major tail gene with the head morphogenesis genes did not come as a surprise since a rho-independent terminator was predicted downstream of ORF 202. This terminator separates the major tail gene from the remainder of the other tail genes. Interestingly, the transcription separation of the tail genes ORF 202 and 117a in Sfi21 occurs at a genome position where Sfi21 deviates substantially from the otherwise closely followed gene map of lambdoid phages (Brüssow and Desiere, 2001). ORF 117a "replaces" two lambda genes (G and T) and no evidence for translation frameshift was obtained This suggests a distinct mode of tail morphogenesis in phage Sfi21 when compared to lambdoid coliphages. In addition to a putative transcription start site upstream of the portal gene, primer extension analysis revealed supplementary putative transcription start sites upstream of the proteinase and the major tail genes. This indicates some flexibility in the expression of the head morphogenesis genes. All these 5′ ends were followed by a ribosomal binding site. In contrast, the major head protein, probably the most abundant protein produced during phage Sfi21 infection, is neither preceded by a putative transcription start site or by a ribosomal binding site. The major head protein is proteolytically processed (Desiere *et al.,* 1999), possibly by the ClpP-like protease encoded by the gene upstream of the major head gene. The transcription and translation of the head gene might thus be coupled to the expression of this protease.

Outlook

Our analysis has proven that *in silico* analysis can provide a relatively reliable first orientation on the genetic organization of a phage genome. The detection of synteny and the conservation of gene order within the lambda supergroup of *Siphoviridae* allowed gene predictions even when no informative database matches were obtained. Transcription mapping is a suitable tool that can verify these *in silico* predictions. In addition, the analysis of the temporal transcription pattern provides a dynamic picture of the phage genome and allows insight into the regulatory circuits. To fully exploit this approach, the viral genome expression must be studied under various conditions of phage–cell interaction. Here we described the transcription pattern of a temperate *S. thermophilus* phage under lytic infection conditions. The transcription analysis of phage Sfi21 under distinct conditions (establishment/maintenance of lysogeny, induction of a lytic infection cycle from the prophage state) could shed light especially on the role of the genetic switch region in this temperate phage. It will also be interesting to compare the observed lytic transcription

pattern to that obtained with the closely related virulent *S. thermophilus* phages and deletion/replacement derivatives of phage Sfi21.

MATERIALS AND METHODS

Bacterial strains, bacteriophages, and media

The *E. coli* strain XL1-Blue was cultivated aerobically in LB broth or on LB broth solidified with 1.5% (w/v) agar at 37°C. *Streptococcus thermophilus* strain SfiI and transformants thereof were routinely subcultured at 42°C in either LM17 (M17 supplemented with 0.5% lactose) or Belliker (DIFCO, Detroit, MI) media. Phage Sfi21 was propagated on *S. thermophilus* strain SfiI in LM17 broth. Phage enumeration was achieved by plaque assay as described by Foley *et al.* (1998).

Construction of plasmids

The cloning vector used in this study was the high copy number *E. coli*/lactococcal/streptococcal shuttle vector pNZ124 (Platteeuw *et al.,* 1994). A 2534-bp *Xba*I fragment from the lysogeny module of the *S. thermophilus* phage Sfi21 was cloned in the *Xba*I site of pUC19 generating the construct pX6. The insert was then transferred as a *Bam*HI–*Hin*dIII fragment from pX6 and cloned in the respective sites of pNZ124 generating the construct pSFswi. The putative cI-like repressor gene, ORF 127, was cloned independently of ORF 75, the putative *cro* repressor gene, by inserting the 664-bp *Bsr*BI/*Bam*HI fragment of pX6 in the *Ecl*136II/*Bam*HI sites of pNZ124, generating the construct pSFcI. pSFcro consists of a 517-bp *Bam*HI/*Xba*I PCR fragment (containing the putative genetic switch region, ORF 75, and the 5′ ends of ORF 127 and 287) cloned in the respective sites of pNZ124. The PCR fragment was generated using phage Sfi21 DNA as the template and primer pair 1F/R (5′-GG CTGCAGGATCCG ACA TGG AAC TGT TGT C-3′ and 5′-GC TCTAGA AAG TTC ATT CAT CGT CA-3′, containing *Bam*HI and *Xba*I restriction sites, respectively).

DNA techniques

DNA samples were amplified in a Perkin–Elmer thermal cycler programmed for 30 cycles each consisting of 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min. Synthetic primers were designed according to the established phage Sfi 21 DNA sequence (Accession No. NC_000872) and used together with the relevant DNA template and *Taq* polymerase Fementas. PCR products were purified using the Qiagen PCR-purification kit following the instruction of the supplier.

Total RNA isolation

Batch cultures (350 ml) were prepared by inoculating Belliker broth with 3.5 ml of an overnight culture of *S. thermophilus* SfiI followed by incubation at 42°C until an OD_{600nm} of 0.2 was reached. $CaCl_2$ was added to a final concentration of 10 mM before phage Sfi21 was added at a multiplicity of infection of 8.

Samples (20 ml) were taken at 0, 2, 7, 17, 22, 32, and 37 min after the start of infection. Immediately after collection, the cell samples were frozen in a ethanol–dry ice bath.

Total RNA was isolated by resuspending the frozen bacterial cell pellets in 1 ml of TRIzol Reagent (GibcoBRL, Gaithersburg, MD), adding 106 μm glass beads (Sigma Chemical Co., St. Louis, MO), and shearing the slurry with a Mini-Beadbeater-8 cell disruptor (Biospec Products, Bartlesville, U.K.) as described by Walker *et al.* (1999). Standard procedure to minimize RNase contamination was used (Sambrook and Russel, 2001).

Northern blot analysis of phage transcripts were carried out on 15 μg aliquots of total RNA separated on a 1.5% agarose-formaldehyde denaturing gel, transferred to a zeta-Probe blotting membrane (Bio-Rad Laboratories, Richmond, CA) by the method of Sambrook and Russel (2001) and fixed by UV cross-linking using a Stratalinker 1800 (Stratagene).

Northern blots and hybridizations

Prehybridization and hybridization of the Northern blots were carried out at 65°C in 0.5 M $NAHPO_4$ (pH 7.2)–1.0 mM EDTA (pH 7.0)–7% sodium dodecyl sulfate (SDS). Following 18 h of hybridization, the membrane was rinsed twice (30 min) at 65°C in 0.1 M NaHPO4 (pH 7.2)–1.0 mM EDTA–1% SDS, twice (30 min) at 65°C in 0.1 M $NaHPO_4$ (pH 7.2)–1.0 mM EDTA–0.1% SDS, and exposed to X-OMAT autoradiography film (Eastman Kodak Co., Rochester, NY).

The probes for the Northern blot hybridization were labeled with [α^{32}P] by using the random-primed DNA labeling system (Boehringer Mannheim GmbH) and purified with Nuc Trap probe purification columns (Stratagene). Their location on the phage genome are provided in Fig. 1.

Primer extension analysis

The 5′ ends of RNA transcripts were determined in primer extension reactions conducted with 15 μg of total RNA mixed with 1 pmol of primer (IRD800 labeled) and 2 μl of Buffer H (2 M NaCl, 50 mM PIPES, pH 6.4). The mixture was denatured at 90°C for 5 min and then hybridized for 1 h at 42°C. After addition of 5 μl 1 M Tris–HCl (pH 8.2), 10 μl 0.1 M DTT, 5 μl 0.12 M $MgCl_2$, 20 μl 2.5 mM dNTP mix, 0.4 μl (5 U) reverse transcriptase (Sigma), and 49.6 μl double-distilled water, the enzymatic reaction was incubated for 2 h at 42°C. The reaction was stopped by adding 250 μl ethanol/acetone mix (1:1) and incubated at −70°C for 15 min followed by a centrifugation at 10,000 rpm for 15 min. The pellets were dissolved in 4 μl distilled water and mixed with 2.4 μl loading

TABLE 1

Primer Sequences Used

Oligonucleotide	Position[a]	Sequence 5′ → 3′
110	23074–23053	CCTTCTTTAGCATCAGCCCAAG
65	23577–23602	GATATAACTCATATAGAAAGCGCAG
359	25547–25568	CAGCAGCCTTGATAGCTTCTG
203	26282–26303	GCAATATAGAATGAAACATTG
122-a	26497–26520	CGTTTTTCACAAAAGTCTCATCAC
122-b	26716–27740	GGTAAATCCCTA TTGATGTAAACG
127	27111–27130	CCCAGCTTTA TTTGCTAATTCTG
75	27498–27477	CCATTCTCTCTCTTGTTGTAC
287	27749–27726	GAAGTTTTG TTCAACCCATTGAC
443	30529–30508	CAATGTGAGCCATCACCACGG
170	37296–37273	CTACTGATTTTACTGGTTCCATG
157	29368–29346	CATAATCACTAGTCCAGTCAATC
504-a	33548–33526	GGGGCGTTCTCAACTAATTCTG
143	34881–34858	GCGTTGCATTGCACATCTATATG
132	39685–36678	GACGTCACTTTAAGCGCTTGTG
175	40171–40154	GTGCAATGGTCTCTCTAC
152	167–147	CGATTTTGCGCCATGTTTCTC
623	744–720	CGTTACC TTGTCTTTGTAAGTCTC
623-2	1772–1751	GGGATATACGAAA CCAAGAGC
623-3	1527–1505	CTTGTTCCAAATCTAAAAGCGG
623-4	2003–1982	GACAACGTCAAGTGAGTTGTC
59	2536–2519	CCAATGCACACCAAACC
384	2735–2712	CCCATTCACTACCATTCAAGGCG
221	3781–3792	GAAGTCCTTATCATCATTGCTTAC
397	4563–4544	GCTTGCA ATTCTTCAGCAC
202	7244–7219	CATTTTGCGATTACGCCGTCTGTTG
117	7951–7932	CTTGTGTGTCGCTTCCCTC
1560	8502–8481	CTTTCTTGAGCCTTCCAAGCG
515	13164–13140	GTTGATGCTATGTTTCTCACAAC
1276	14738–14712	CAAATGTACTTGTTGAGTATTCAAGG
670	18545–18523	GCTAATCGTAACCTAACTTGAC
141b	21106–21084	CGCTTCCAATCATTTCAATCAG

[a] The bp positions refer to the Sfi21 sequence reported in NC_000872.

buffer from the sequencing kit (Thermo-sequenase fluorescence labeled, Amersham). The cDNA was separated through 8% polyacrylamide–urea gels along with sequencing reactions which were conducted using the same primers employed for primer extensions and detected using the LiCor sequencer machine (MWG Biotech).

The synthetic oligonucleotides (MWG Biotech) used for primer extension experiments were listed in Table 1.

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